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THE RÔLE OF PHAGOCYTOSIS IN THE ANTHRACIDAL ACTION OF DOG BLOOD.*

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INTRODUCTION.

THE demonstration by Wright and Douglas¹ of the exact interaction between serum and cells in phagocytosis makes it feasible to study the part played by leucocytes in the destruction of certain bacteria *in vitro*. The pathogenic bacteria most suitable for this purpose are such as are not destroyed by lysins in the serum, e. g., streptococci, pneumococci, and anthrax bacilli (especially so far as dog blood is concerned). The mechanisms of invasion and of healing in infections by these organisms present many difficult problems in the efforts at solution of which special attention must be given to the leucocytes, because of the prominent part played by them in the reactions of the infected body. G. F. Ruediger² has shown that opsonin and leucocytes are essential to destruction of streptococci by human blood, and in this communication I propose to trace the rôle of phagocytosis in the anthracidal action of dog blood.

THE OPSONIN FOR ANTHRAX BACILLI IN DOG SERUM.

Dog leucocytes are actively phagocytic *in vitro* for anthrax bacilli in the presence or under the influence of normal dog serum. When leucocytes—i. e., blood corpuscles or the leucocytes in pleural exudates—are washed carefully many times in NaCl solution, so as to remove all traces of serum, they no longer take up normal bacilli. The phagocytic power of washed leucocytes is restored by adding normal dog serum, but the action of the serum is not exercised directly on the leucocytes, but upon the bacilli, because bacilli that have been treated with normal serum and then washed in NaCl solution are taken up by washed leucocytes—by absorbing the

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¹ *Proc. Roy. Soc.*, 1903, 72, p. 357, and 1904, 73, p. 128.

² See this number p. 156.

opsonin of the serum the bacilli become susceptible for phagocytosis. The opsonin for anthrax bacilli in dog serum is diminished greatly by heating the serum at 56° C. for 30 minutes, and as a rule,* wholly or almost wholly destroyed by heating at 60° C. for the same length of time.

The facts just stated are illustrated in Table I, which gives the results of some of the experiments.

TABLE I.
PHAGOCYTOSIS OF ANTHRAX BACILLI BY DOG LEUCOCYTES.

| BLOOD 0.5+BACILLARY SUSPENSION 0.5 | PHAGOCYTOSIS (200 LEUCOCYTES COUNTED) | |
|---|--|-----------------|
| | Phagocytosis | No Phagocytosis |
| Defibrinated blood | 192 | 8 |
| Washed blood | 10 | 190 |
| " " +normal serum | 190 | 10 |
| " " +serum heated at 56° C. 30 min | 60 | 130 |
| " " + " 60° C. 30 " | 30 | 170 |
| " " +bacilli sensitized in normal serum | 130 | 70 |
| " " + " " " serum heated at 56° C. 30 min | 44 | 156 |
| " " + " " " " 60° C. 30 " | 4 | 196 |

It is quite impossible to count the number of anthrax bacilli taken up by leucocytes; a good idea of the extent of phagocytosis is obtained, however, by counting a number of leucocytes and noting the relation between those that are and those that are not engaged in phagocytosis.

Considerable phagocytosis takes place within 15 minutes after adding bacilli to defibrinated dog blood, and gradually increases during the first hour or two. Within the leucocytes the bacilli appear to lose their power to take up stains, and disappear as shadows and irregular fragments, just as described by Metchnikoff many years ago. Often the bacilli are arranged parallel to one another in the leucocytes. Leucocytes also appear to stretch themselves along the anthrax thread, and by contracting curl up the latter within themselves. In the presence of normal serum and bacilli the leucocytes engaged in phagocytosis manifest a distinct tendency to form groups, which is not the case at all in mixtures of normal bacilli and washed leucocytes.

* Individual variations occur undoubtedly. In experiments of this kind the leucocytes used must be carefully washed free from serum.

In order to sensitize bacilli for phagocytosis a small loopful of a 24 hour agar culture is suspended in 1 c.c. of NaCl solution to which is added 1 c.c. of serum. The suspension is placed at 37° C. for 30 to 60 minutes, when the bacilli are centrifugated out, washed, and resuspended in NaCl solution. Frequently only 50 or 60 per cent of leucocytes are found engaged in phagocytosis in the case of bacilli sensitized in normal serum. This may mean that the union between opsonin and bacillus is easily destroyed and part of the opsonin lost in the washing.*

DESTRUCTION OF ANTHRAX BACILLI BY DOG LEUCOCYTES.

The question now arises whether it is possible to show by test-tube experiments to what extent, if any, phagocytosis of anthrax bacilli by dog leucocytes is associated with destruction of the bacilli. I believe that the results of the experiments recorded in Table 2 point very directly to the conclusion that phagocytosis and destruction of anthrax bacilli by dog leucocytes *in vitro* go hand in hand. In these experiments one loopful of the various suspensions of anthrax bacilli used was added to the different mixtures. By plating another loopful in each case, and counting the colonies that had developed at the end of 24 hours, the approximate total number of bacilli added was obtained, and this is the number given in the column headed "at once." Two sets of the different mixtures were made, each tube of which received one loopful of anthrax suspension; by plating the whole quantity in each tube, those of one set at the end of two or three hours, and those of the other set at the end of five hours, and then counting the colonies at the end of 24 hours, an approximately correct idea of the fate of the bacilli introduced was secured (see columns in Table 2 headed "2 hours" and "5 hours").

* It will be seen, too, from the other tables that the number of colonies is not reduced so greatly when sensitized bacilli are added to washed blood, as when normal bacilli are mixed with defibrinated blood, or with washed corpuscles and normal serum

TABLE 2.
ANTHRACIDAL ACTION OF DOG BLOOD.

| MIXTURES—TOTAL QUANTITY 1 C.C. | NUMBER OF BACILLI IN TOTAL QUANTITY | | |
|---|-------------------------------------|---------|---------|
| | At Once | 2 Hours | 5 Hours |
| Defibrinated blood 0.5 + NaCl sol. 0.5..... | 3,000 | 280 | 20 |
| Washed blood 0.5 + NaCl sol. 0.5..... | 3,000 | 2,750 | ∞ |
| Washed blood 0.5 + normal serum 0.5..... | 3,000 | 181 | 114 |
| Washed blood 0.5 + NaCl sol. 0.5 + bacilli treated with normal serum..... | 3,120 | 628 | 105 |
| Washed blood 0.5 + NaCl sol. 0.5 + bacilli treated with serum heated at 56° 30 min..... | 980 | 1,962 | ∞ |
| Washed blood 0.5 + NaCl sol. 0.5 + bacilli treated with serum heated at 60° 30 min..... | 1,540 | 2,036 | ∞ |
| Washed blood heated at 45°c. 0.5 + normal serum 0.5..... | 3,500 | 6,475 | ∞ |
| Dog serum 0.5 + NaCl sol. 0.5..... | 3,692 | 6,590 | ∞ |
| Serum 0.5 + NaCl sol. 0.5 + bacilli treated with normal serum..... | 1,600 | 3,500 | ∞ |
| Broth 0.5 + NaCl sol. 0.5..... | 3,092 | 11,550 | ∞ |
| Broth 0.5 + NaCl sol. 0.5 + bacilli treated with normal serum..... | 3,076 | 9,860 | ∞ |

(The sign ∞ means innumerable.)

Table 2 shows that normal anthrax bacilli grow freely in suspension of washed dog blood, in dog serum, in suspensions of washed dog corpuscles containing serum heated for 30 minutes at 56° and 60° C.; and in normal serum and washed dog blood heated at 45° for 30 minutes (there is no phagocytosis in such mixtures); further, that bacilli sensitized in the manner described in normal serum multiply in dog serum and in broth, as do also bacilli sensitized in serum heated at 56° and 60°, and then added to suspensions of washed corpuscles. Note that the corpuscular suspensions concerned in these cases are suspensions in which no or little phagocytosis takes place, as shown in Table 1. Destruction of normal bacilli takes place in defibrinated blood and in washed blood plus normal serum; destruction also takes place when bacilli sensitized in normal serum are added to suspensions of washed dog leucocytes.

When smaller numbers of bacilli (500 to 1,500), normal or sensitized, are added to defibrinated blood or to suspensions of leucocytes respectively, complete sterility may be obtained at the end of three and five hours. When the mixtures are agitated moderately in a shaking machine there is usually a greater destruction than when they stand quietly, probably because of the better opportunity for phagocytosis in the former instance.

The essential rôle of the corpuscles—leucocytes—in the destruction of anthrax bacilli by dog blood becomes very distinct in plates

made with decreasing quantities of blood and a fixed quantity of bacilli, the total quantity being made up to 1 c.c. in all cases by the addition of normal dog serum. As shown in Table 3, the de-

TABLE 3.

DIMINISHING DESTRUCTION OF ANTHRAX BACILLI WITH DECREASING QUANTITIES OF DOG CORPUSCLES (LEUCOCYTES) AND INCREASING QUANTITIES OF DOG SERUM.

| QUANTITY MADE UP TO 1 C.C. BY ADDING NORMAL DOG SERUM | NO. OF BACILLI IN TOTAL QUANTITY | | |
|---|----------------------------------|---------|---------|
| | At Once | 3 Hours | 5 Hours |
| Defibrinated blood 1 c.c. | 1,400 | 0 | 0 |
| " " 0.75 c.c. | " | 79 | 560 |
| " " 0.5 c.c. | " | 89 | 700 |
| " " 0.25 c.c. | " | 840 | 3,500 |
| " " 0.125 c.c. | " | 1,750 | 4,650 |
| " " 0.062 c.c. | " | 1,600 | 10,000+ |

struction decreases as the amount of corpuscles decreases, in spite of the fact that there is a compensatory increase in the amount of serum. While the majority of these experiments have been made with dog blood, a sufficient number of experiments has been made with pleural exudates of dogs to indicate plainly that the same results are obtainable with the leucocytes and serum of exudates as with those of the blood, both as regards bacteriolysis (Table 4)

TABLE 4.

DIMINISHING DESTRUCTION OF ANTHRAX BACILLI WITH DECREASING QUANTITIES OF WHOLE EXUDATE FROM PLEURAL CAVITY OF DOG AND INCREASING QUANTITIES OF SERUM.

| QUANTITY MADE UP TO 1 C.C. BY ADDING SERUM OF EXUDATE | NO. OF BACILLI IN TOTAL QUANTITY | | | |
|---|----------------------------------|---------|-------------------|---------|
| | Virulent Bacilli | | Avirulent Bacilli | |
| | At Once | 5 Hours | At Once | 5 Hours |
| Exudate 1 c.c. | 3,000 | 43 | 3,220 | 15 |
| " 0.75 c.c. | " | 46 | " | 6 |
| " 0.5 c.c. | " | 17 | " | 27 |
| " 0.25 c.c. | " | 58 | " | 148 |
| " 0.125 c.c. | " | 148 | " | 1,050 |
| " 0.062 c.c. | " | 700 | " | 910 |
| " 0.03 c.c. | " | 1,750 | " | 1,330 |
| Washed leucocytes from exudate 1 c.c. | " | 4,700 | " | 3,290 |
| Serum of exudate 1 c.c. | " | 7,350 | " | 9,170 |

and phagocytosis. It is evident that in different experiments comparable results are obtainable only when the same quantity and quality of leucocytes are employed, granting that other conditions are equal.

Defibrinated blood of newborn dogs destroys anthrax bacilli to

just as great extent as that of adult dogs. In one experiment 4,500 bacilli were added to 0.5 c.c. of defibrinated blood of a dog five days old; in two hours the number of bacilli was reduced to 29; in four hours the plates gave 220, and in six, 2,590. In this case the polymorphonuclear leucocytes seemed unusually active in phagocytosis, practically every cell being crowded with bacilli.

THE OPSONIN AND AMBOCEPTOR FOR ANTHRAX BACILLI IN
DOG SERUM NOT IDENTICAL.

I have shown that the element—opsonin—in dog serum that prepares the anthrax bacilli for phagocytosis by dog leucocytes, and without which phagocytosis does not take place, is practically destroyed by heating serum at 60° C. for 30 minutes. Consequently the anthrax opsonin in dog serum is probably not identical with the amboceptor for anthrax bacilli that Bail and Petterson¹ have shown is present in the serum of dogs. These investigators have demonstrated that, while dog serum does contain an amboceptor that unites with anthrax bacilli, these, nevertheless, are not destroyed by dog serum, because the serum does not contain the proper complement. Rabbit serum, however, does contain complement that in the presence of dog serum destroys anthrax bacilli. In accord with Bail and Petterson, I find that minute quantities of rabbit serum,* in themselves without appreciable anthracidal action, may render otherwise harmless dog serum markedly anthracidal.

Now, I have found further that heating dog serum to 60° C. for 30 minutes destroys its power to render mixtures of dog serum and dog leucocytes anthracidal, but it does not interfere with its destructive action on anthrax bacilli when complemented with rabbit serum as shown in Table 5.

TABLE 5.
SUCCESSFUL COMPLEMENTATION OF DOG SERUM HEATED AT 60° C. BY NORMAL RABBIT SERUM.

| TOTAL QUANTITY 1 C.C. | NO. OF BACILLI IN TOTAL QUANTITY | |
|---|----------------------------------|---------|
| | At Once | 4 Hours |
| Dog serum heated at 60° C. 30 min. 0.5 + normal rabbit serum 0.1..... | 1,240 | 3 |
| “ “ “ “ “ “ “ 0.25..... | “ | 14 |
| Normal rabbit serum 0.1 | “ | 10 |
| “ “ “ 0.05 | “ | 4 |
| “ “ “ 0.025 | “ | 3,200 |
| | | 3,200 |

¹ *Centralbl. f. Bakt.*, 1903, 33, pp. 343, and 610; *ibid.*, p. 613.

* There is considerable individual variation in the anthracidal power of normal rabbit serum.

TABLE 6.
ACTION OF RABBIT SERUM UPON ANTHRAX BACILLI TREATED WITH DOG SERUM.

| NO. OF BACILLI ADDED | NO. OF BACILLI IN TOTAL QUANTITY IN 3 HOURS | | |
|--|--|----------------|-----------------|
| | 1 C.C. RAB. SER. | 0.05 RAB. SER. | 0.025 RAB. SER. |
| Bacilli treated with normal serum—480..... | 0 | 80 | 168 |
| " " " serum heated at 56°—370 | 0 | 33 | 54 |
| " " " " " 65°—656 | 0 | 96 | 46 |
| Normal bacilli—440 | 4 | 1,000 | 1,066 |

Table 6 shows in a little different manner that the amboceptor in dog serum is not demonstrably affected by heating serum at 56° and 65° C. for 30 minutes, because bacilli treated in serum so heated are destroyed by quantities of rabbit serum so minute as to have in themselves no demonstrable lytic action upon normal bacilli. Under these circumstances it is reasonable to conclude that the anthrax opsonin and the anthrax amboceptor in dog serum are distinct bodies, and, inasmuch as dog serum, when heated at 60° C., loses its opsonin, and with it the normal power to render the bacilli destructible by washed dog corpuscles—a power possessed by normal dog serum—that the amboceptor in dog serum plays no at present demonstrable rôle in the destruction of anthrax bacilli by dog serum and corpuscles, i. e., polymorphonuclear leucocytes. And this must be so in spite of the fact that when anthrax bacilli are treated with dog serum, they probably unite with both opsonin and amboceptor.

THE ANTHRACIDAL SUBSTANCE IN DOG LEUCOCYTES.

When large quantities of dog leucocytes, obtained from aleuronat exudates, are carefully washed many times, and then extracted with sterile distilled water or allowed to undergo autolysis under toluol, a clear fluid may be secured which is destructive of anthrax bacilli. Similar leucocytic extracts have been obtained by Petterson¹ and others. The anthracidal property of the extracts that I obtained has been quite resistant to heat. There is at present no evidence to indicate that this substance, whatever it may be, plays any appreciable rôle outside of the leucocytes in the test-tube experiments recorded in this paper, because I have invariably ob-

¹ *Centralbl. f. Bakt.*, 1905, 39, p. 423.

tained a multiplication of anthrax bacilli inoculated into suspensions of washed leucocytes in NaCl solution.

SUMMARY.

Anthrax bacilli are destroyed in normal defibrinated dog blood owing to the combined action of the serum and the phagocytes.

The substance in the serum necessary for phagocytosis and destruction of the bacilli is destroyed by heat at 56° – 60° C. for 30 minutes, and it is probably not identical with the anthrax amboceptor present in dog serum.

The destructive action of dog leucocytes on anthrax bacilli in the presence of, or influenced by, normal dog serum is suspended by previously heating the leucocytes at 45° for 30 minutes, when phagocytosis no longer takes place.

Washed dog leucocytes contain a thermostable, anthracidal substance, extractible by distilled water and autolysis, but there is no evidence to show that this substance has any effect outside of the cells in the usual test-tube experiment.

Phagocytosis is an essential step in the destruction of anthrax bacilli by dog leucocytes and dog serum.